Impact of presence of excipients in drug analysis in fed-state gastric biorelevant media

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Abstract: In this study, the influence of the presence of excipients in sample preparation and clean-up steps required prior to drug analysis in milk-based media which simulate the *in vivo* properties of the fed state stomach was investigated. 15 excipients, normally present in solid dosage forms of five APIs tested (atenolol, paracetamol, furosemide, nifedipine and propafenone hydrochloride) were mixed (one at a time) with the active pharmaceutical ingredient of interest either via vortexing, co-grinding or shaking of the physical mixture and dissolved in Fed State Simulated Gastric Fluid (FeSSGF). The objective of the study was the assessment of the extraction efficiency of three protein precipitation protocols (using MeOH, ACN and 10% w/v TCA), typically used in drug analysis, in milk-based biorelevant media in the presence of the excipients. The mixing technique, fat content of the medium and excipient and solvent effects were investigated. The efficiency of three different protein precipitation reagents in drug extraction when dissolved as API:excipient mixtures in the fed-state medium was compared against the equivalent drug amount recovered in the absence of the excipient in FeSSGF. Most excipients had a significant negative effect ($p < 0.05$) on drug recovery in the milk-based medium as indicated by the multiple linear regression (MLR) analysis performed. For magnesium stearate and HPMC, the % recovery values were the lowest in four out of the five drugs studied, with a range of 10-100% depending on the API, mixing technique and protein precipitation protocol selected. The negative excipient-dependent effect was more profound in nifedipine and propafenone hydrochloride, the most lipophilic compounds of the study. Acetonitrile was the most effective extraction reagent for most drugs in the presence of excipients, followed by methanol and 10% w/v trichloroacetic acid. Data analysis also revealed a dependence of the extraction method efficiency on the medium lipid content. Application of the above extraction protocols in commercially available formulations highlighted the need for assessment of the effect of excipients in extraction efficiency, before transferring the method directly to dissolution studies of formulations in milk-based fed gastric media. In conclusion,

the presence of excipients and the selection of protein precipitation protocol are parameters which can affect significantly the efficiency of protein precipitation when FeSSGF is used as dissolution medium and need to be taken into consideration when developing a quantitative method based on the above sample clean-up technique.

Keywords: Fed state, Protein precipitation, Excipients, Formulation, Biorelevant media, Drug analysis

Abbreviations: **ACN**:acetonitrile, **API**:active pharmaceutical ingredient, **BCS**:biopharmaceutics classification system, **FeSSGF**:fed state simulated gastric fluid **FeSSGF$_{hf}$**: fed state simulated gastric fluid prepared with high fat milk (5% w/v), **FeSSGF$_{sk}$**:fed state simulated gastric fluid prepared with skimmed milk, **IVIVC**:*in vitro-in vivo* correlation, **MeOH**:methanol, **MLR**:multiple linear regression, **VIF**:variance inflation factor, **rpm**:revolutions per minute, **TCA**:trichloroacetic acid

1. Introduction

Poor drug solubility has always been one of the biggest challenges the pharmaceutical industry has had to overcome. Approximately 40% of marketed drugs are classified as poorly soluble, while 90% of the drugs entering the screening process during drug development have poor solubility according to the Biopharmaceutics Classification System (BCS) [1]. Therefore, different formulation strategies were employed in order to improve the solubility and dissolution characteristics of the active pharmaceutical ingredients (APIs) and also act as carriers of the active substances, contributing, among others, to the formulations' stability, appearance, biopharmaceutical profile, manufacturability and patient acceptability [2-4].

A second challenge concerns the development of media used for drug *in vitro* dissolution testing. Dissolution tests, as dictated by the United States Pharmacopoeia (USP) [5], cannot always provide information on the behaviour of the drugs *in vivo*, and therefore the need for multi-phase dissolution media able to simulate the gastrointestinal environment arose. The employment of media more "biorelevant", targets to simulate the passage of the formulation through the different compartments of the gastrointestinal tract both in the fasted and fed states. The use of such media can contribute towards the correlation of the results of the drug *in vitro* release with its *in vivo* pharmacokinetic performance (IVIVC), with an aim to decrease the number of *in vivo* studies conducted pre- and post-approval [6]. As far as gastric fed environment is concerned, the media developed could be classified in two categories: i. milk-based media, such as full-fat milk, digested milk and Fed State Simulated Gastric Fluid (FeSSGF) and ii. Lipid emulsions, such as Ensure®, Ensure Plus® and Intralipid® [7]. The media of the above two categories were developed as an attempt to simulate the gastric environment after the administration of a high or low fat standard breakfast respectively [8]. The composition of such media is constantly updated [9] and takes into consideration the protein/carbohydrate/lipid ratio and content as well as the fed gastric physicochemical

properties measured *in vivo* [8, 10], making reasonable compromises. A disadvantage in the use of such media concerns their treatment prior to the sample analysis, which usually requires laborious steps for the extraction of drug before its quantification. Steps which may include the precipitation of the medium's proteins, extraction through drug partition between a mobile liquid phase and a non-miscible liquid solvent or a solid stationary phase, centrifugation and filtration are typically required prior to drug analysis.

When a formulation is dissolved in such media, interactions may be formed between the excipients (excipient-excipient interactions) and also between the excipient and the active ingredient (excipient-drug interactions) or the dissolution medium (excipient-medium interactions). Not only can such interactions affect the solubility and dissolution rate of the drug in biorelevant media, but they can also play a role in the design of sample clean-up techniques, the effectiveness of which may be compromised if only designed based on the physicochemical properties of the APIs. Therefore, an appropriate design would elucidate the role of excipients in the analysis in heterogeneous biorelevant media, such as the milk-based media used for the simulation of the gastric fed state in dissolution studies, with the current study focusing on the last two types of interactions. The type of interactions between drugs and excipients are described as physical or chemical [11], depending on their ability to induce chemical changes in the drug or excipient. Binding of drugs which have primary amine functional groups in the molecule to microcrystalline cellulose is a typical example of a physical interaction, leading to drug entrapment in the cellulose [12]. Primary amines can also interact with double bonds of certain excipients, like sorbitan monooleate via a reaction analogous to Michael addition, which is considered a chemical interaction. Changes in the excipient behavior, as a result of their interaction with the heterogeneous gastric environment, have been characterised a challenge which needs to be addressed in drug dissolution and analysis [13]. A known example of interactions between excipient and medium involves

polyvinylpyrrolidone, which can form hydrogen bonds with water molecules through its carbonyl group [14], and undergo phase separation in aqueous 1.5 M potassium fluoride solutions. In cases where the milk-based or lipid emulsion-gastric biorelevant media of interest are used in drug dissolution studies, such medium-excipient interactions may be even more complicated. The formation of protein and fat gel layers around hydroxypropyl methylcellulose (HPMC) matrices, which could potentially affect drug extraction when nutrient drinks or milk are used as dissolution media, is a typical example of medium-excipient interaction [15, 16].

In the present study, we investigated the impact of the presence of excipients in drug extraction when dissolved in the milk-based gastric fed state biorelevant media. Three hydrophilic (paracetamol, atenolol, furosemide; log P < 2) and two moderately lipophilic (propafenone hydrochloride and nifedipine; log P 2-4) drugs were selected as model compounds. The excipients selected for the study consist of binders, lubricants, extended release matrix agents, emulsifiers, wetting agents and disintegrants. HPMC is the most common cellulose used in hydrophilic matrices. It is used as a binder and also provides extended release characteristics to oral dosage forms [17, 18]. Magnesium stearate and stearic acid are used as tablet and capsule lubricants, avicel (microcrystalline cellulose) as binder and lubricant and povidone as binder, diluent and coating agents [18]. Tween 80 and sodium dodecyl sulfate (SLS) are employed as emulsifying, solubilizing and wetting agents [18]. Polyethylene glycols have various uses, such as suspending agents, co-solvents, binders, plasticizers or lubricants, depending on their solid state and molecular weight [18]. Croscarmellose sodium is used as disintegrant in tablets and capsules and Eudragit L100 and Eudragit E are brand names for polymethacrylate copolymers used as drug coatings for enteric drug delivery and taste masking respectively [19]. Finally carbomer 974P is used as a binder and also as suspending, gelling and emulsifying agent [18]. Drug recovery in commercially available formulations was also assessed. The impact of excipients in drug analysis in a fed

gastric medium was analysed using a regression analysis method [multiple linear regression (MLR)]. The study is a follow up of a previous study [20], where the impact of the active ingredients' physicochemical properties (log P, ionisation, aqueous solubility and protein binding) on the extraction protocol from FeSSGF media (protein precipitation and solid phase extraction) was assessed. Twenty active substances, including the five drugs of the current study, were used as model drugs in order to assess the effect of the above physicochemical properties on the applicability of a number of extraction protocols from drugs dissolved in the middle FeSSGF version in the absence of excipients. Extractions with a variety of organic and aqueous reagents of different volumes, different SPE cartridges and elution solvents were performed. The optimum protocols, as determined in the presence of active substance only, are employed in this piece of work and their transferability in API:excipient mixtures is assessed. The objective of this work is the investigation of the impact of excipients in the efficiency (expressed as percentage of drug recovered) of the protein precipitation protocols, developed and optimized for the analysis of the APIs. Except for the drugs' physicochemical properties, the current work aims to assess the dependence of drug-excipient mixing technique, protein precipitation extraction method, and dissolution medium's lipid content in an attempt to provide further insight towards the optimization of drug analysis in fed state media, and the analytical methods' application in formulations.

2. Materials and methods

2.1. Materials

APIs: Furosemide (≥ 98% (HPLC)) and propafenone HCl (≥ 98% (HPLC)), were purchased from Sigma- Aldrich, UK. Nifedipine (98.0-102.0% (on dried substance)), paracetamol (97.5% min. (HPLC)) and atenolol (≥ 98% (TLC)) were purchased from Fisher Scientific, UK. **Excipients:** SLS (≥ 99.0%) (GC)), povidone K30 (meets USP testing

specifications), PEG 400 (202398), HPMC (H7509), were purchased from Sigma- Aldrich, UK. PEG 300 (Eur Pharm), stearic acid ((≥ 99.0%) (GC)) and PEG 6000 were purchased from Merck Millipore, UK. Microcrystalline cellulose (Ph-302) and croscarmellose sodium, (NF, Ph. Eur., JP) were purchased from FMC Biopolymers, UK. Carbomer 974P (Carbopol), PEG 4000 and magnesium stearate (Ph.Eur., BP, USP) were purchased from Fischer Scientific, UK. Tween 80 was purchased from VWR. Eudragit E (powder) and Eudragit L100 were purchased from Evonik Industries, UK. **Formulations:** Adalat® LA 30 mg tb (Bayer, UK), Arythmol® 300 mg tb (Abbot Healthcare, UK) **Filters:** Cronus 13 mm regenerated cellulose (RC) syringe filters 0.45 µm were purchased from LabHut Ltd, UK. Whatman 13 mm glass microfiber syringe filters 2.7 µm (GF/D) were purchased from Fisher Scientific, UK.

Sodium acetate trihydrate, hydrochloric acid (36.5-38%), glacial acetic acid (≥ 99%), trichloroacetic acid 10% w/v and all phosphate salts were purchased from Fisher Scientific, UK. HPLC grade methanol, acetonitrile, trifluoroacetic acid (≥ 99.0%) and formic acid were all purchased from Sigma- Aldrich, UK.

0, 3.6 and 5% fat UHT milk was commercially purchased (Sainsbury's, UK).

2.2. Instrumentation

All samples were analysed in an HPLC system consisting of an Agilent 1200 series binary pump (G1312A), an Agilent 1200 series DAD detector (G1315D), an Agilent 1200 series autosampler (G1329A), an Agilent 1200 series controller (G1316A) with a Chemstation software (Agilent Technologies, Santa Clara, United States).

A pH meter Mettler-Toledo AG (model SevenCompact pH/Ion S220, Schwerzenbach, Switzerland), a centrifuge Hereus Biofuge Primo R (Thermo Scientific, Hanau, Germany) and a vortex mixer Rotamixer (HTZ, Cheshire, UK) were used.

2.3. Medium selection and preparation

Fed State Simulated Gastric fluid (FeSSGF) was selected as the working fed state medium due to its simplicity in its preparation. Its buffer capacity, osmolality and surface tension values are overall closer to the values measured *in vivo* after the administration of a standard meal than the equivalent properties of milk, which has been extensively used as a gastric fed state medium in dissolution studies [21]. FeSSGF was prepared according to Jantratid et al [9] by mixing 3.6% fat milk and acetate buffer pH = 5 at a 1:1 volume ratio. pH was adjusted to 5 with 1 N HCl. Apart from the standard version, two other different versions of FeSSGF were prepared, using 0% w/v ($FeSSGF_{sk}$) and 5% w/v ($FeSSGF_{hf}$) fat milk.

2.4. Selection of drug-excipient combinations

Assessment of the impact of excipients on % recovery in FeSSGF was conducted for five drugs (atenolol, paracetamol, furosemide, nifedipine, propafenone hydrochloride) selected from the study which involved the optimisation of extraction protocols for APIs of a wide range of lipophilicity, ionisation and aqueous solubility [20]. Drug working concentration was defined as the usual recommended administered single drug dose dissolved in 500 mL of medium, unless limited by the solubility of drug in FeSSGF (Table 1). In the cases where drug solubility in FeSSGF was the limiting factor, the concentrations obtained from drug solubility studies (24 h) conducted in FeSSGF [20] were used. The current study assessed the effect of 15 excipients commonly used in commercial formulations of the above drugs (Table 2), using the optimised protein precipitation protocols for the quantification of active pharmaceutical ingredients developed in the previous study [20]. In summary, the extraction protocol involves addition of 2 parts of either MeOH, or ACN or 10% w/v TCA in 1 part of medium, brief vortexing (30 sec), centrifugation [(8000 rpm (11400 g), 15 min, 4 °C)] and filtration through a regenerated cellulose 0.45 μm filter. The sample was diluted with “blank” acetate buffer pH

5 or MeOH:acetate buffer 1:1 pH 5, according to the drug solubility, where peak shape needed to be improved. The excipients used were selected based on the ones present in their commercial formulations as given in the Electronic Medicines Compendium (eMC) [22]. The percentage of the drug in the mixture was arbitrarily set as 30% w/w of the formulation.

Extraction efficiency was given by drug (theoretical) absolute % recovery, expressed as:

$$\%\ \text{absolute recovery} = \frac{\text{amount of drug in drug-excipient mixture FeSSGF solution}}{\text{amount of drug in FeSSGF drug solution}}$$

where the amount of drug in the presence of excipient was quantified against calibration standards of the drug in FeSSGF. The amount of drug in the absence of excipient was treated and analysed using the same methodology as the respective drug-excipient mixture (treated with the same protein precipitation reagent).

2.5. Assessment of drug-excipient mixing process, type of medium and formulation analysis

The effect of the drug mixing process was assessed in a pilot study using three (atenolol, paracetamol and propafenone) of the five drugs. The mixing method of choice was selected on the basis of method robustness and lower data variability. An appropriate quantity of drug powder (40 mg atenolol, 40 mg paracetamol, 16 mg furosemide, 12 mg nifedipine, 120 mg propafenone hydrochloride), equivalent each working concentration, was mixed for 3 minutes with one related excipient at a time by either i. Using mortar and pestle, ii. Manual shaking in an Eppendorf tube or iii. Vortexing in an Eppendorf tube. API:excipient ratios were selected based on the percentage of each excipient in commercial formulations (Table 2), within the range dictated in the "Handbook of Pharmaceutical Excipients" [18]. PEG 300 and PEG 400, which are liquid, were mixed with the drug by manual shaking in a volumetric flask for 3 min before the addition of FeSSGF. A quantity of drug-excipient mixture, containing an amount of

drug equivalent to the working drug concentration (Table 1) was transferred in a 200 mL volumetric flask and filled with FeSSGF. The flasks were incubated at 37 °C in a shaking water bath (200 shakes per min) for 90 min. Three 1 mL samples were taken from the top of each volumetric flask, filtered through GF/D filter, and each was treated with a different protein precipitation reagent (methanol, acetonitrile, 10% w/v TCA) as explained in the study for the extraction of the active pharmaceutical ingredients previously conducted [20]. The samples were filtered through 0.45 μm filters and were analysed using HPLC. Amber flasks were used for nifedipine mixtures with all experiments conducted in a light-protected environment. The autosampler, centrifuge tubes and water bath were covered in foil and amber vials were used for the HPLC analysis of nifedipine.

The same process was performed in the high fat ($FeSSGF_{hf}$) and no fat ($FeSSGF_{sk}$) versions of FeSSGF, for the drug-excipient mixtures so as to assess the effect of the medium lipid content in drug recovery. Extractions in the above media were conducted in the mixtures initially exhibiting drug recovery < 50% in FeSSGF (MeOH used as extraction solvent).

Commercial nifedipine and propefenone hydrochloride formulations (Adalat® LA tb 30 mg and Arythmol® tb 300 mg) were each placed in glass bottles filled with 500 mL of FeSSGF and were incubated at 37 °C under strong agitation (200 rpm) for 48 h so as to achieve maximum drug release. A sample was taken from each flask, filtered through a GF/D syringe filter and drug was extracted with the same precipitation reagent used for the physical mixtures and analysed as explained above. All experiments were performed in triplicate and % recovery was expressed as mean ± standard deviation.

Adsorption studies were performed in triplicate for each model drug for all types of filters used for each drug-solvent combination. A standard solution prepared in extraction solvent (MeOH, ACN, 10% w/v TCA): acetate buffer pH 5, 2:1 was filtered through the RC

and GF/D filters. The first ten drops of the filtrate were discarded and the concentration of the aliquot of the remaining filtrate was measured using HPLC. Two consecutive filtrations were performed. No adsorption issues were observed for the drugs studied (adsorption < 5%) [23]

2.6. HPLC analysis

Stock solutions of propafenone hydrochloride, nifedipine, atenolol, furosemide and paracetamol were prepared in MeOH. Calibration standard solutions were prepared in FeSSGF, $FeSSGF_{hf}$ and $FeSSGF_{sk}$. The drugs were analysed in HPLC with the analytical methods (modification of published methods) used specified in the table below (Table 3).

Adsorption studies were performed in triplicate for each model drug for all types of filters used. No adsorption issues were observed for the drugs studied.

2.7. Statistical Analysis

% absolute recovery and correlation with excipient type, mixing method and protein precipitation reagent used in drug analysis were evaluated in the context of a multi-way Analysis of Variance (ANOVA) with a post-hoc Bonferroni test (Statgraphics® v. XVI, StatPoint Technologies Inc, US). Comparisons where $p < 0.05$ suggested a statistically significant difference.

The % recovery data was analysed via multiple linear regression (MLR) so as to investigate the impact of the selected excipient using SPSS v.22.0 (SPSS®, Chicago, IL, USA). Interactions of selected excipients with drug lipophilicity (log P) were included in the model on the basis of the lowest % recovery observed for the specific excipients (HPMC and magnesium stearate) in the pilot study. The generated MLR models were evaluated in terms of their regression coefficient (R^2) and variance inflation factor (VIF) with high R^2 values referring to a good fit to the model and VIF values < 3 indicating absence of multicollinearity

among the independent variables [24]. The standardized coefficients of the factors plotted indicate the relative positive/negative effect on their corresponding values. The importance of each factor was evaluated by its p value. Statistical significance was set at $p < 0.05$.

3. Results and Discussion

3.1. Effect of drug-excipient mixing technique on % drug recovery in milk-based media (pilot study)

The contour graphs for the three model drugs of the pilot study express the % drug recovered as a function of type of excipient and mixing process (Fig. 1a-c) for every protein precipitation reagent (MeOH, ACN, 10% w/v TCA) used for their extraction. It can be observed that % recovery was affected in all three drugs; an effect denoted by the colour change across the mixing process axis (y axis). For the three compounds selected, the mixing process and the physicochemical properties of the drug substance had a significant effect on their % recovery after the medium's protein precipitation (Fig. 2).

Effect of mixing technique

Vortexing and grinding gave significantly different % recovery values ($p < 0.05$) in all three drugs (Fig. 2), which implied that the powders' handling may affect their homogenous mixing. In both hydrophilic drugs (paracetamol and furosemide), grinding process led to lower recovery values than in the other two mixing processes (Fig. 2).

Grinding is a common strategy used to reduce drug particle size, often applied to drugs of poor solubility with an aim to increase their bioavailability *in vivo* [25]. Micronisation via grinding increases the drug's specific area, leading to enhanced dissolution [26] according to the Noyes-Whitney equation. Improved dissolution is attributed to reduced particle size and consequently higher surface area and better contact of the micronised drug with the dissolution

medium [27]. Although co-grinding of drugs with several excipients, such as lactose monohydrate [28] and avicel [29] accelerated their dissolution profile, there are cases when co-grinding may be used to prolong dissolution and lead to a sustained release profile [30]. A study with theophylline, a hydrophilic compound with log P value similar (log P = -0.02 [31]) to the above drugs, demonstrated that co-grinding with magnesium stearate decreased the dissolution efficiency and mean dissolution rate of the formulation [32] in comparison to a physical mixture of the same powder quantities. Therefore, a reason for the decreased drug % recovery when an excipient more hydrophobic than the active ingredient and the drug are co-ground could possibly be the concentration of hydrophobic particles of decreased particle size around the API, leading to delayed drug dissolution in the fed state medium. Moreover, micronized substances can lead to higher particle agglomeration due to higher cohesion and consequently development of electrostatic charging and poor flowability properties which can affect drug handling [27].

The differences in % recovery observed between manual shaking and vortexing in the two most hydrophilic drugs can possibly be attributed to the formation of mixtures of different homogeneity; manual shaking is possibly unable to provide the mixing force required, leading to the formation of areas with increased or decreased drug concentration in the mixture, while vortexing could potentially lead to the entrapment of particles of lower density on the walls of the centrifuge tube creating similar homogeneity issues.

Effect of the active substance's properties

As observed in Fig. 1a, the % recovery of paracetamol was close to 100% in the vast majority of cases with all reagents, excipients and mixing methods (mean paracetamol recovery = 100.9 ± 9.6%), a fact possibly attributed to paracetamol's increased wettability. Contact angle measurement is a method of determination of the wettability of a compound, with angles > 90° being indicative of poor compound wettability with the dissolution medium [33]. The measured

327 contact angles of paracetamol's polymorph commercially used, monoclinic form one [34],
328 against water ranged from 15.9° (± 3.1°) to 67.7° (± 2.5°) depending on the crystal facet [35],
329 which is indicative of increased wettability with the aqueous part of the medium. Higher
330 wettability can lead to enhanced dissolution rate in the medium and consequently higher
331 recovery due to the increased amount of drug solubilized in the medium. Therefore, despite
332 paracetamol's high polarity and lower ability to associate with fats in the medium, recovery
333 close to 100% was obtained by using all three mixing techniques. Recovery values > 100%
334 can be attributed to the mixing limitations of the techniques selected and also to the inherent
335 variability of the sample preparation due to the extraction, centrifugation and filtration steps.
336 The % recovery values of furosemide (Fig. 1b) ranged between 40.9 and 110.2% depending on
337 the excipient and extraction reagent used for drug recovery. Water's contact angle against
338 furosemide has been measured > 90° and could provide a reason for the lower recovery values
339 compared to paracetamol [36]. The effective surface area for a specific drug substance in a
340 solvent has been associated with the powder's wettability, given by the contact angles in the
341 respective solvent [37]. The wetting of a soluble agglomerate though is a multiple step process
342 (initial wetting of the power, penetration of the liquid between different part of agglomerate,
343 immersion into the solvent and dissolution of the particles forming the agglomerate) which
344 cannot easily be described by a single model. It is therefore difficult to associate differences in
345 the dissolution of the drug-excipient mixtures (and consequently differences in the amount of
346 drug recovered) with specific effects in each of above steps caused by the drug's or excipient's
347 properties [38]. The extraction values of propafenone hydrochloride, a lipophilic drug, were
348 lower than in the two hydrophilic drugs described above (Fig. 1c, 2), and had a wider range. %
349 recovery values were as low as 17 ± 7.9% (HPMC/shaking mixing method/drug extracted with
350 10% w/v TCA). In this case, grinding led to significantly higher drug recovery values than in
351 the other two mixing methods, despite the fact that some of the excipients were common in the

other drugs as well (HPMC, magnesium stearate), which suggests that the extraction efficiency in the milk-based medium depends both on the mixing process and API's properties.

The decrease in particle size and the possible alteration of the powders' surface properties when ground with mortar and pestle were two parameters which had to be taken into consideration in assessing the effect of excipients and protein precipitation solvent. Therefore, vortexing was selected as the powder mixing method for the assessment of the above parameters in all drugs and excipients. Despite showing equally high deviation between samples with the others it was more easily controlled than manual shaking (constant stirring speed) and provided homogenous mixing with minimal changes of the powders' surface.

3.2. Effect of excipient type and extraction reagents (protein precipitation solvents) on drug % recovery in milk-based media

The protein precipitation results of the full study (mixtures of the 5 drugs with the 15 excipients) are presented in Fig. 3, with the drug % recovery expressed as a function of combined excipient and drug. Drugs are sorted by increasing log P values from bottom to top. Multiple linear regression analysis of the results of the full study, in combination with the multiple comparisons of the pilot study were used to evaluate the effect of the excipients and protein precipitation solvent on % drug recovery. Overall, the results of the MLR analysis of the drug-excipient mixtures for the extraction of each protein precipitation solvent showed good fits with high R^2 values (0.76, 0.66 and 0.7 for the extraction with MeOH, ACN and 10% w/v TCA respectively) and VIF values < 3 for the independent variables of the model (Fig. 4). The analysis demonstrated statistical significance for most of the variables tested ($p < 0.05$).

3.2.1. Excipient effect in drug recovery from milk-based media

The pilot study performed with the three drugs, revealed that the % recovery is both API and excipient dependent. The multiple comparisons' test performed with the three drugs

showed that in all cases, their mixtures with magnesium stearate and HPMC led to significantly different recoveries compared to the rest of the drug-excipient mixtures, with lower mean % recovery values (Fig. 2). When the excipient effect was evaluated for all five drugs (vortexing), decreased % recovery values were observed in more drug-excipient mixtures in addition to the two mentioned above, denoted by the blue and green zones in the contour plots of the vortexed mixtures (Fig. 3). Furthermore, most excipients showed a statistically significant negative effect on % drug recovery, as demonstrated by the results of the MLR analysis (Fig. 4), with the excipient effect discussed for each drug separately below. From the contour map of the full study (Fig. 3), it can be observed that the recovery values for the three hydrophilic drugs (atenolol, paracetamol, furosemide) were distinctively higher than in the mixtures of the lipophilic ones (nifedipine, propafenone hydrochloride).

In the case of paracetamol, none of the excipients decreased % drug recovery more than 15% except for HPMC, which was attributed to the drug increased wettability as reported above. When HPMC was mixed with other active ingredients as well, (atenolol, nifedipine, propafenone hydrochloride) the % recovery of the drugs ranged from approximately 80 % for paracetamol down to approximately 20% for propafenone hydrochloride, regardless of the extraction solvent of choice (Fig. 3). In the cases of HPMC and magnesium stearate for atenolol, recovery values as low as 61.3 and 56.9% were observed for the two excipients respectively. In furosemide mixtures, the drug recovery was mainly controlled by the extraction reagent, rather than the excipient. ~~an effect discussed in the section below~~. One possible reason for the lower recovery in the presence of HPMC could be the formation of a barrier of fat and/or proteins formed around the powder in the milk-based medium, decreasing medium permeation [39] to the inner part of the formulation, as reported in several studies where nutrient drinks like Ensure Plus® or Nutrison® were used as fed gastric dissolution media [15] [40]. It was also demonstrated that the initial gel formation layer of HPMC matrices during dissolution

increased according to the fat percentage of the fed state medium, although the difference could be attributed to other properties of the medium, such as its viscosity [16]. Magnesium stearate acts by preventing the adhesion of the powder during tablet compression, forming a non-uniform hydrophobic layer on the surface of the powder mixture [41, 42]. Therefore, the decreased % recovery values in all drugs mixed with magnesium stearate could be attributed to the slower drug dissolution in the medium, due to the excipient's hydrophobic nature [43]. Interestingly, although most excipients demonstrated a significant negative effect on drug % recovery (green bars), and HPMC and magnesium stearate showed a negative effect in the multiple comparisons test of the pilot study, they did not have a statistically significant contribution to the MLR final model (Fig. 4). Their interactions with drug lipophilicity though demonstrated a highly significant effect, as shown by the high standardized coefficients of the respective variables (HPMC*log P, magnesium stearate*log P) (Fig. 4). In the case of propafenone, a more lipophilic drug, drug recovery in the presence of HPMC and magnesium stearate was significantly lower than in the presence of the other excipients ($p < 0.05$) (Fig. 2). % recovery values were the lowest in the presence of the former, and did not exceed 40% regardless of the choice of the extraction solvent. The challenging nature of the extraction of lipophilic drugs from HPMC matrixes has been reported and attributed to the high API lipophilicity and the gelation properties of the polymer [44], an effect demonstrated in the current study too, as indicated by the high negative HPMC*log P standardised coefficients in MLR analysis (Fig. 4). Therefore, the negative impact of HPMC and magnesium stearate on drug recovery is more profound in drugs of high lipophilicity (log P) and can attributed to the possible formation of layers around the drug powder either self-induced or in combination with the milk-based medium [15, 41, 42].

A similar explanation could be given for the negative effect of povidone K30, a hydrophilic polymer which has been shown to increase both wettability and dissolution rate of

lovastatin, a drug of similar lipophilicity to the lipophilic drugs (nifedipine, propafenone hydrochloride) of the study [45] (log P = 4.26 [31]).

As far as the effect of the excipient on the dissolution of the lipophilic drugs is concerned, the short duration of the study (90 min) seems to be the most probable reason for the log P/excipient-dependent % recovery. Most of the excipients appear to have a log P-dependent effect on the dissolution of the APIs, meaning that a time > 90 min would be required for the total amount of lipophilic drugs to be solubilized. The combinations of nifedipine with all excipients demonstrated % recovery values < 80%. Except for the HPMC and magnesium stearate, the effect of which on drug dissolution was previously described, the lowest values reported for nifedipine were in its mixtures with Eudragit L100, Eudragit E and carbopol 974P. Eudragit L100 is insoluble below pH 6, according to the manufacturer, which could result in co-precipitation of the drug in the FeSSGF of pH= 5. According to the excipients' product characteristics, Eudragit polymers are highly soluble in polar organic solvents and 1N HCl, which indicates that the above excipients are more likely to act as recovery enhancers rather than prohibitors. ~~Eudragit E is soluble at gastric fluid of pH up to 5, meaning that in the working pH, after the addition of methanol or acetonitrile, precipitation of the excipient along with the drug could take place due to the pH of the supernatant (pH of the supernatant measured > 5).~~ The reason of the decreased drug recovery values when APIs were mixed with carbopol 974P can be attributed to the same reason as HPMC and magnesium stearate; the formation of a gel layer around the particles of the active ingredient decreasing the diffusion coefficient of the drug in the medium [46]. Another possible mechanism suggesting reduced drug transport to the dissolution medium due to interaction between the drug and the polymer has also been reported in the literature [47]. Avicel's negative effect on nifedipine recovery (< 60% with all protein precipitation reagents) (Fig. 3) could also be attributed to the entrapment of the smaller drug particles between the microcrystalline cellulose's particles leading to slower drug wetting

and dissolution [48]. Water soluble polyethylene glycols (PEG 4000, PEG 6000) and Tween 80 were normally expected not to affect % drug recovery negatively, as they act as solubility enhancers [49] and surfactants [50], improving the solubility and dissolution characteristics of poorly soluble drugs. The effect of PEG 300 and SLS in atenolol recovery was minimal (> 79.5% in all mixtures), while the % recovery in the presence of avicel, croscarmellose sodium and magnesium stearate were all approximately 60, 80 and 100% when 10% w/v TCA, MeOH and ACN were used for the extraction of drug from FeSSGF respectively (Fig. 3).

Despite the different impact of each excipient on drug recovery, the study design did not account for the effect of absolute excipient and drug concentrations in the working solutions. Hence, the drug-excipient effect was assessed as an entity, with the drug:excipient ratio being the constant parameter in all cases. Despite the representative ratios used in the study, to the best of our knowledge, keeping a different variable constant such as the active substance or concentration could potentially lead to different conclusions.

3.2.2. Protein precipitation reagent effect in drug recovery from milk-based media

For the three hydrophilic drugs (atenolol, paracetamol, furosemide), recovery values > 60% were observed for all three reagents (Fig. 3), with the highest recovery observed in ACN extraction, as the red zones of the graph (Fig. 3) indicate. In regard to the lipophilic drugs (nifedipine, propafenone hydrochloride), the amounts of drug extracted were distinctively lower for all three reagents. Particularly for 10% w/v TCA, the percentage of drug recovered in the presence of excipients was extremely low; less than 40% of the drug was recovered in 15 of the total 16 nifedipine/propafenone hydrochloride and excipient mixtures (Fig. 3). At the TCA concentrations used, unfolding of the medium's proteins is set off by negatively charged ions of the acid, which cause the disruption of the electrostatic forces maintaining the structure of its proteins. This mechanism of action can potentially expose non-polar protein surfaces and lead to coalescence of the molecules and precipitation [51]. It is possible that the presence of

476 excipients may inhibit the solubilisation of the drugs by the acid and facilitate its occlusion in
477 the precipitate.

478 The effect of the type of protein precipitation solvent was statistically significant in
479 terms of % recovery ($p < 0.05$), as demonstrated by the ANOVA analysis of the pilot study
480 (Fig. 2). The differences can be observed in the different reagent levels of the visual ANOVA
481 representation (Fig. 2) and also in the different colour zones of the contour graphs (Fig. 3); the
482 reddest zones observed belong to ACN and the bluest to 10% w/v TCA, indicating the highest
483 and lowest recovery values respectively. The above order of extraction efficiency (ACN >
484 MeOH > 10% w/v TCA) was followed in four of the five drugs regardless of excipient of
485 mixing method, except for atenolol as observed in the contour plots (Fig. 1, 3). In the case of
486 atenolol mixtures, which was the only drug more poorly extracted with ACN than with TCA,
487 the better applicability of trichloroacetic acid cannot be directly justified by the drug's
488 physicochemical properties, as in the absence of excipient both ACN and 10% w/v TCA were
489 able to recover approximately 100% of the drug in FeSSGF [20]. It could be suggested that
490 drug's comparatively higher solubility in trichloroacetic acid than in acetonitrile [52] led to
491 faster drug solubilisation in its mixture with the excipient. The similarity in the contour patterns
492 and the standardized coefficients of MeOH and ACN in Figures 3 and 4, respectively, and their
493 dissimilarity to those of TCA, highlight the impact of solvent strength and polarity on drug
494 recovery, which is expected and highly dependent on the compounds' non-polar characteristics.

495 It is worth mentioning, that the extraction efficiency (in terms of effective drug
496 recovery) of the three reagents used in the presence of excipients, followed the same pattern as
497 in their absence, as demonstrated in a previous study [20]. In both studies, drug lipophilicity
498 had a negative effect on the amount of drug recovered, which may suggest that the presence of
499 excipients may amplify the differences in drug recovery, previously correlated with the drug's
500 physicochemical properties. It should be clarified that the negative effect of lipophilicity is

attributed to the drug's decreased dissolution rate in the medium, rather than the effect of the excipient in extraction efficiency. This is also supported by a previous study in drug extraction in FeSSGF [53], which did not show any statistically significant correlations between drug lipophilicity and sample treatment efficiency using MeOH or ACN. Studies using liquid extraction techniques as a means for sample treatment prior to analysis in milk have demonstrated the effect of solvent polarity in drug recovery [54]. and the importance of solvent miscibility with the aqueous phase and their salting-out potential [55].

Therefore, the physicochemical properties of the API need to be taken into consideration in the design of an effective sample clean-up method, both in the absence and presence of excipients.

3.3. Effect of medium fat content on drug %recovery in milk-based media and assessment of drug extraction method in solid oral dosage forms

Analysis in media of different fat content was performed for the two lipophilic drugs of the study, nifedipine and propafenone hydrochloride, in mixtures with excipients demonstrating the low (< 50%) drug % recovery (magnesium stearate, carbomer 974P, Eudragit L100 and HPMC).

The recoveries in the high and no fat media for the excipient mixtures with nifedipine were equally low. Nifedipine recovered ranged from approximately 3 to 16% and from 4 to 18% for the low and high fat medium, while the equivalent values for FeSSGF gave values from 7 to 50% for the three reagents used (Fig. 5). The reduced values in the low fat medium, compared to FeSSGF, were attributed to the lower drug solubility. Nifedipine's solubility in FeSSGF is approximately 70 μg/mL [56]; a value approximately 7 to 10-fold higher than the drug's aqueous solubility, with the difference possibly attributed to the solubilisation of the lipophilic drug in the lipid portion of the medium. Therefore, the absence of fat in the medium

525 may be the reason for the lower drug solubility and consequently lower recovery. In the high-
526 fat medium, low recovery values are possibly attributed to the decreased extraction efficiency
527 in media of high fat content, a common issue of sample clean-up methods in milk-based media
528 [7]. A study of HPMC-nimodipine mixtures in acetate buffer pH = 4.5 (log P = 3.41 [57],
529 similar structure and lipophilicity to nifedipine) showed an increase in the drug solubility and
530 dissolution efficiency by a factor of 4 compared to the drug in the absence of the excipient [58].
531 The above points out that low solubility and dissolution rates cannot always be attributed to
532 the excipient but to the interactions between the mixture and the dissolution medium as well.
533 In the case of propafenone, medium prepared with skimmed milk ($FeSSGF_{sk}$) gave higher drug
534 recovery values when ACN and TCA were used as protein precipitation reagents (Fig. 5),
535 which could be attributed to the difficulty of designing an effective extraction protocol in
536 media of high lipid content. Propafenone, as a lipophilic compound was expected to have
537 higher solubility in the full fat medium, due to its preferential distribution in its lipid portion.
538 Lower recovery can possibly be attributed to the decreased extraction strength of the solvents
539 due to the drug's distribution to the medium's fat.

540 Extraction of drug of commercial formulations revealed that despite the presence of the
541 excipients, their extraction was in no case affected to such an extent as with the simple mixing
542 of each excipient separately. The % recovery value was in all cases between 67.2 and 99.5%
543 (Fig. 5a, b) and its dependence on the solvent selection was evident, in the same way as with
544 the physical mixtures of APIs and excipients. Acetonitrile was most effective, followed by
545 methanol and 10% w/v TCA. The reagent-dependent recovery results in drug formulation
546 indicate that suitability of the extraction method for the active ingredient does not necessarily
547 guarantee equivalent extraction performance in formulation analysis. The reduced recovery in
548 extraction with trichloroacetic acid may indicate entrapment or adsorption of the drug in
549 excipients, and inability of the method to break these excipient-drug interactions. A use of such

a method without prior assessment of drug-excipient interactions could lead to erroneous results if potentially used for drug quantification in a dissolution study using milk-based media. The differences in recovery values among formulations and reagents, along with the results of the assessment of the mixing technique, confirmed that the process via which drugs and excipients are formulated has to be considered for the development and optimisation of a sample clean-up protocol. As the differences reported between drug-excipient mixtures point out, the conclusions of the current study are not directly transferable to the commercial formulations. Excipients during formulation development undergo several changes resulting from the nature of the manufacturing process. An increase in the tablet's surface area as a result of direct compression [59] or formation of granules of different size, porosity and friability [60] may lead to different drug dissolution behavior in fed state media. However, good knowledge of the excipients effect in drug analysis could potentially be used in the optimisation of sample treatment and drug analysis in the process of formulation development when excipients are one at a time, or in formulations in which the excipients undergo minimal changes in terms of their physicochemical characteristics (e.g oral solutions).

4. Conclusions

The presence of excipients can have an effect on the amount of drug extracted from milk-based gastric media and should therefore be taken into consideration when developing a quantitative method for drug analysis. Using a previously developed extraction protocol [20], the effect of excipients used in commercial formulations in the recovery of drugs when dissolved in milk-based fed gastric media was investigated. The results demonstrated dependence of the type of excipient, mixing technique and protein precipitation protocol selected with the interaction between lipophilicity and certain excipients (HPMC, magnesium stearate) being highly influential in most cases, as indicated by the MLR analysis. The

differences in the impact of the same excipients in drugs of different lipophilicity highlighted the need for further investigation of excipient-drug interactions and the way both the excipients' and APIs' physicochemical properties can affect drug analysis in fed state gastric media. The study revealed a medium-dependent recovery in the presence of excipients, but without indications of a direct correlation between medium's fat percentage and amount of drug recovered. Finally, it was concluded that excipient processing during drug manufacturing may affect the efficiency of the sample clean-up methods and has to be taken into consideration in drug analysis and quantification. Therefore, to accomplish the accuracy required in drug analysis in fed state milk-based media, the effect of drug properties, type of excipient, changes in medium composition and formulation manufacturing have to be individually assessed in regard of their effect on the extraction efficiency. A more complete understanding of the effect of the above characteristics on drug recovery, further testing of a more extensive set of drugs, excipients and reagents could potentially allow the optimization of the sample treatment process in fed media using reduced screening approaches. Further studies which will assess the full applicability of the optimized extraction protocols developed for the active pharmaceutical ingredients on the different types of drug formulations are required.

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5. References

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List of tables

Table 1 Physicochemical properties and working concentrations of model compounds.

Drug	log P [61-65]	pKa [66-70]	Working concentration (µg/ mL)
Atenolol	0.23	9.60	200
Paracetamol	0.46	9.50	200
Furosemide	0.74	3.90	80
Nifedipine	2.91	3.93	60
Propafenone hydrochloride	3.39	9.27	600

Table 2 Mixtures and ratios of excipients and APIs used. "✓" denotes the presence of each excipient and API in the mixture.

	Paracetamol	Furosemide	Propafenone hydrochloride	Nifedipine	Atenolol	Drug:excipient ratio in the mixture
Povidone K30	✓			✓		10:1
HPMC	✓		✓	✓	✓	0.6:1
Microcrystalline cellulose (Avicel)	✓	✓	✓	✓		1.2:1
SLS	✓				✓	20:1
Carbopol 974P				✓		6:1
Eudragit E				✓		3:1
Eudragit L100				✓		3:1
Magnesium Stearate	✓	✓	✓	✓	✓	10:1
Stearic acid	✓					10:1
Tween 80				✓		10:1
PEG 300					✓	10:1
PEG 400			✓			
PEG 4000	✓			✓		
PEG 6000			✓	✓		
Croscarmellose sodium		✓	✓			10:1

[a] Drug: excipient ratio value was selected within the range of % excipient concentration as dictated in "Handbook of Pharmaceutical excipients" [18].

Table 3 HPLC methods (modification of published methods) used for the quantification of the model compounds.

Drug	Column	Mobile phase	Flow rate (mL/ min)	Temp (°C)	Inj. Volume (µL)	λ_{max} (nm)
Nifedipine [71]	Thermo Hypersil BDS C_{18}, 300Å, 250 x 4.6 mm, 5 µm	MeOH:H_2O 60:40	1	20	50	238
Furosemide [72]	Thermo Hypersil BDS C_{18}, 300Å, 250 x 4.6 mm, 5 µm	MeOH:Formic acid 0.1% v/v 60:40	0.8	25	20	233
Paracetamol [73]	Thermo Hypersil BDS C_{18}, 300Å, 250 x 4.6 mm, 5 µm	MeOH:H_2O 20: 80	1	10	20	257
Atenolol [74]	Thermo Hypersil BDS C_{18}, 300Å, 250 x 4.6 mm, 5 µm	MeOH: Phosphate buffer 0.01 M (pH = 4.5) 20:80	1	25	50	240
Propafenone HCl [75]	Agilent Eclipse XDB C_{18}, 120Å, 250 x 4.6 mm, 5 µm	MeOH:ACN: TEA:H_2O 50:7.5:0.1: q.s 100 pH = 2.9	0.8	25	20	248

Figure captions

Fig. 1. Drug-excipient gradient maps for **a.** paracetamol, **b.** furosemide and **c.** propafenone hydrochloride. Contour plot of % recovery values as a function of drug-excipient mixing method and excipient. "Warm" colour regions (yellow, red) indicate that amounts of drug similar to their theoretical recovery values in the absence of excipient (% recovery > 80%) were able to be extracted from the medium, whereas "cold" regions (blue, purple) are indicative of poor % drug recovery.

Fig. 2. Three-way ANOVA of excipient, protein precipitation reagent and mixing method effects (from left to right) on % drug recovery for the three model drugs. Different letters denote statistically different % recovery between excipients, reagents or mixing processes ($p < 0.05$).

Fig. 3. Drug-excipient gradient map (mixed by vortexing). Contour plot of % recovery values as a function of drug and excipient. Red x points denote the drug-excipient combinations used in the mixtures.

Fig. 4. Standardised coefficients of MLR analysis after protein precipitation with **a.** MeOH, **b.** ACN and **c.** 10% w/v TCA. Green colour denotes statistical significance.

Fig. 5 Nifedipine-excipient (**a.**) and propafenone-excipient (**b.**) mixtures, media and commercial formulations gradient map. Contour plot of % recovery values as a function of medium and excipient/formulation. Red x points denote the drug-excipient-medium combination.

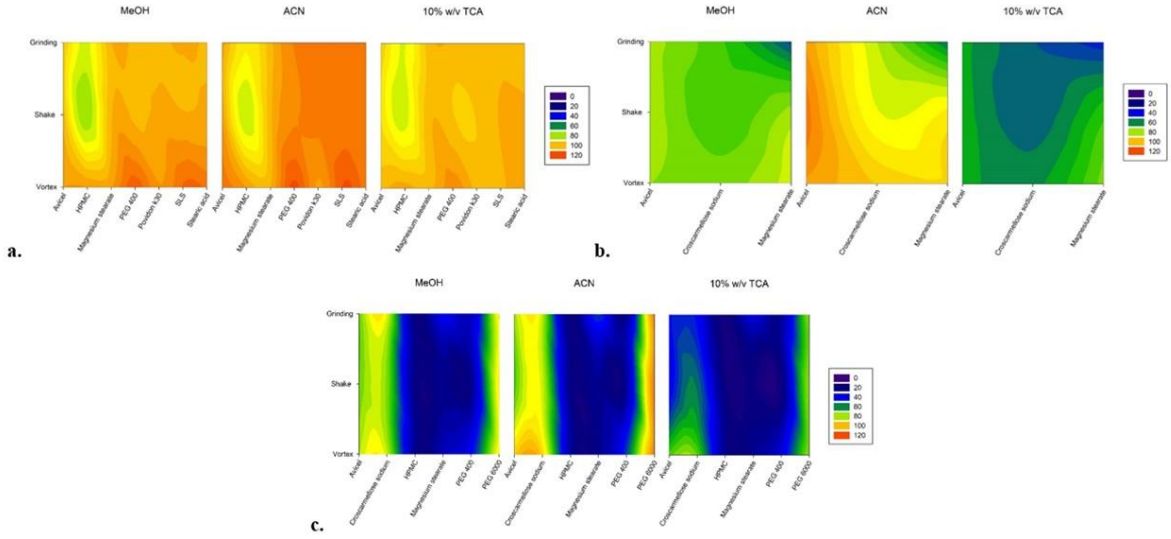

MeOH
ACN
10% w/v TCA
Grinding
Shake
Vortex
0
20
40
60
80
100
120
Avicel
HPMC
Magnesium stearate
PEG 400
Povidon k30
SLS
Stearic acid
Povidon k25
PEG 6000
Crosscarmellose sodium
Magnesium stearate
a.
b.
c.

Figure 1

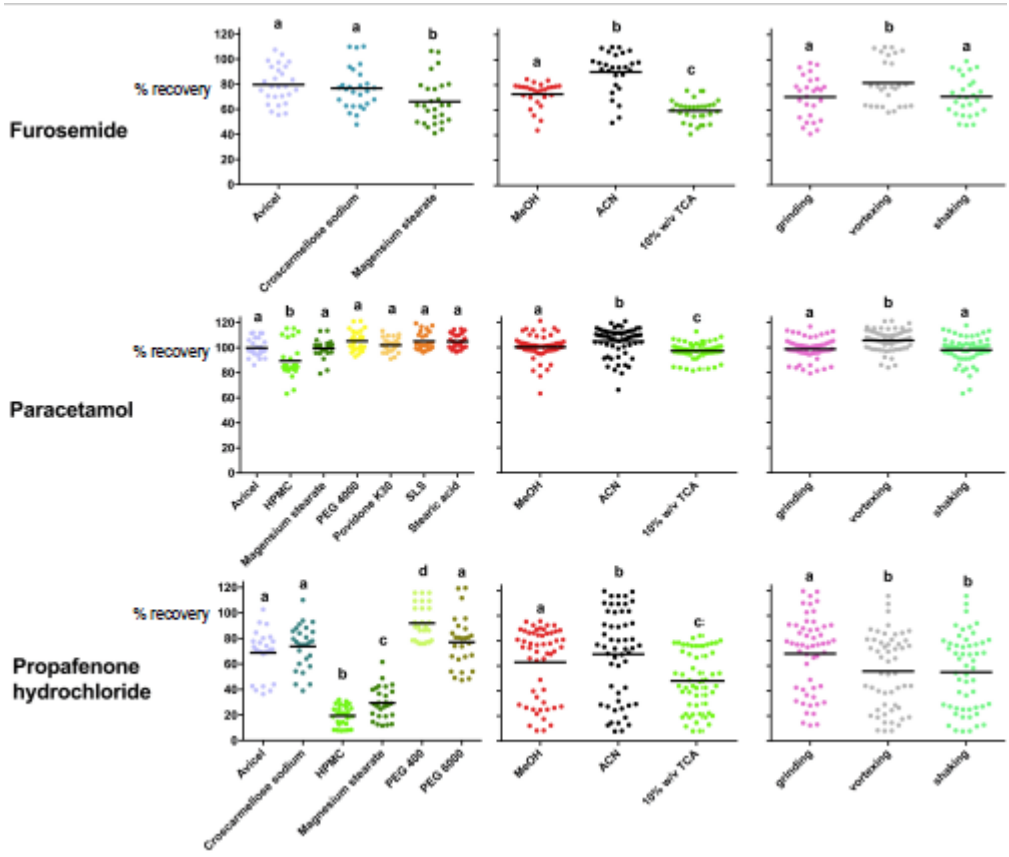
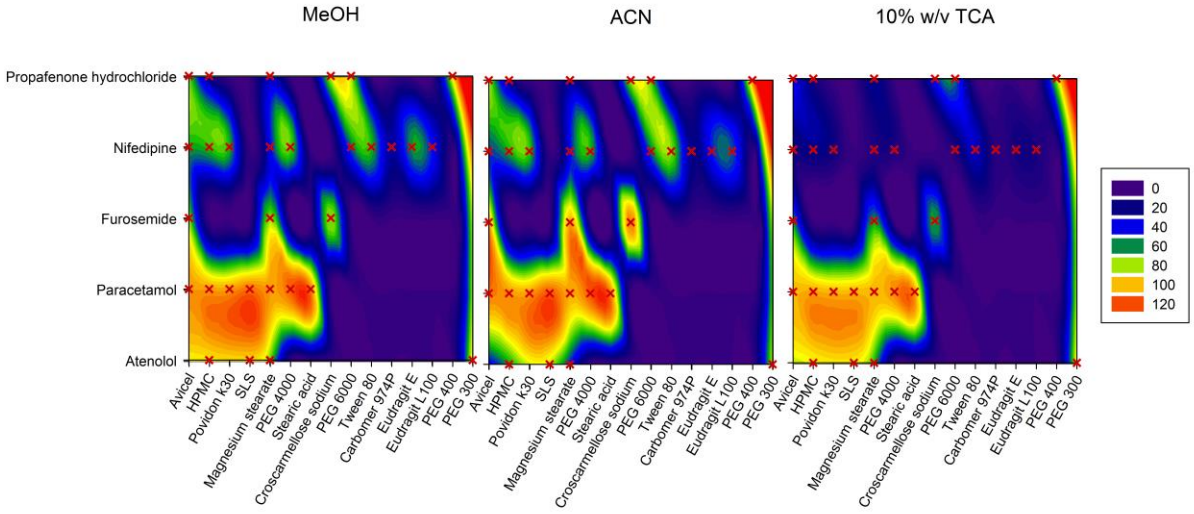

Furosemide
% recovery
Avicel
Croscarmellose sodium
Magnesium stearate
MeOH
ACN
15% w/v TCA
grinding
vortexing
shaking
Paracetamol
% recovery
Avicel
HPMC
Magnesium stearate
PEG 4000
Povidone K30
SLS
Stearic acid
MeOH
ACN
15% w/v TCA
grinding
vortexing
shaking
Propafenone hydrochloride
% recovery
Avicel
Croscarmellose sodium
HPMC
Magnesium stearate
PEG 400
PEG 6000
MeOH
ACN
15% w/v TCA
grinding
vortexing
shaking

Figure 2

840



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842 Figure 3

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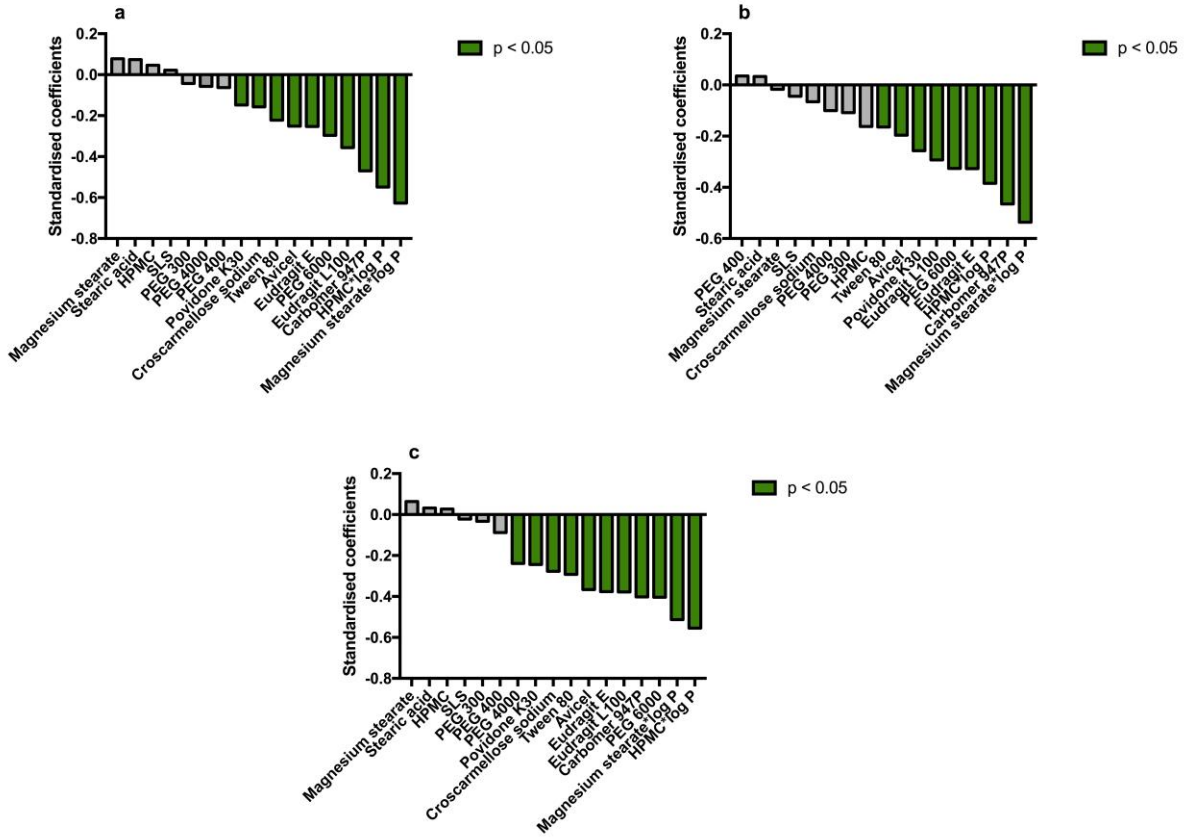

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848 Figure 4

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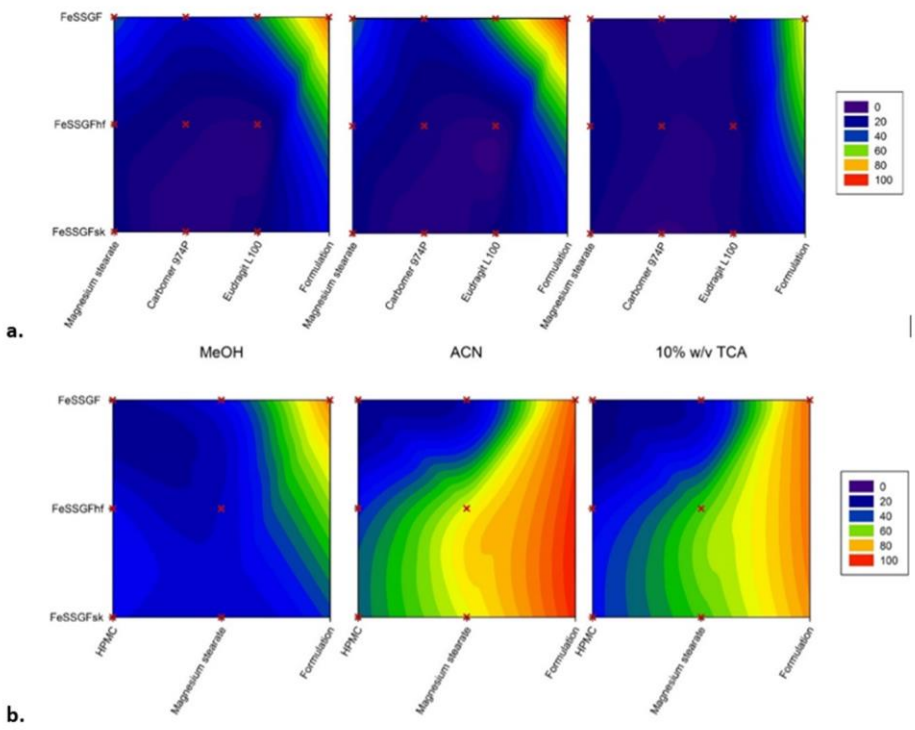
a.
FeSSGF
FeSSGFhf
FeSSGFsk
Magnesium stearate
Carbomer 974P
Eudragit L100
Formulation
MeOH
ACN
10% w/v TCA
0
20
40
60
80
100
b.
HPMC
Magnesium stearate
Formulation

Figure 5